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Clues on the dynamics of DNA replication in *Giardia lamblia*

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MS TITLE: The organization of the Giardia genome explains why part of the population exhibits asynchronous replication between nuclei

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see from their reports, the reviewers' recommendations are mixed. While referees #1 and #2 enthusiastically support publication, referee #3 considers that your study does not reach the standard to be published in JCS. After carefully reading your study, I agree with reviewers #1 and #2 who recommend that a revised version of the manuscript can be reconsidered. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. In particular, you should modify the title as highlighted by reviewer #3.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this paper by M S da Silva et al., the authors investigate the spatio-temporal organization of DNA replication, progression of replication forks, and potential occurrence of head-on replication-transcription collisions in G. lamblia trophozoites using single-molecule techniques, including DNA combing and nanopore-based sequencing.

The authors were able to identify caveats of parasite replication including its high replication rate, long inter-origin distance and the parasite's dependence on a relatively small pool of origins within its genome. Additionally, the authors also show that while roughly 80% of trophozoite stage parasites replicate their nuclei synchronously, about 20% show asynchronous replication between their nuclei.
Overall, the findings are important to understand the replication dynamics and cross-talk with transcription in G. lamblia.

Comments for the author

Comments:
1. Figure 1 records data that was obtained from biological triplicates. However, the histogram in Figure 1.B. showing replication rate frequency does not have error bars. Does this histogram show a sum of all readings obtained from all the replicates ? if so, it would be more accurate to show the mean or median values between replicates with error bars.

2. It is very interesting to see that about 20% of trophozoites in a population have asynchronous replication between their nuclei. Were these parasites synchronized before the double pulse with the halogenated thymidine analogues ? It is not exclusively mentioned anywhere in the manuscript. If not, it would be good to repeat the experiment using cell cycle synchronized parasites. This can be achieved by sorting using FACS or by centrifugal elutriation or chemical arrest-release treatments using nontoxic agents like cycloheximide or low-dose colchicine. Also, the total sample size of 388 cells may not be adequate for a strong statistical correlation. Since this is a qualitative measurement, estimating nuclear patterns in about 1000 cells from three independently synchronized cultures could eliminate bias and provide a more tenable result. Additionally, a non-parametric t-test is required to estimate the significance of this data.

3. The title of the paper implies that the organization of the Giardia genome affects the synchronicity of replication between the parasite's nuclei. However, the paper does not provide a clear correlation/causation for this curious phenomenon. Are there more HoRT collisions in one of then nuclei in cells that are asynchronously replicating nuclei ? If so, a clear correlation is not established here.

4. The observations recorded in the paper are under normal healthy conditions. While this is useful, it would be more appropriate to study this under conditions that simulate replication stress. Would the asynchronicity in replication between the two nuclei increase in propensity under nutrient limiting conditions ? Would a low dose of DNA damaging agents like Cisplatin or Methyl methanesulfonate exacerbate it ? Would reactive oxide stress from peroxides have an effect on it? In order to justify the title of the manuscript, such conditions might provide some context as to why there is synchronicity in replication between the two nuclei and what can disrupt it.
It would be useful to study this phenomenon in context to the localization and abundance of a replication protein like PCNA or a polymerase or a replication fork licensing/initiating protein. This would help to quantitate the asynchronicity between the two nuclei in terms of the enrichment of these proteins, and might help to identify a pathway that regulates synchronicity in replication between the two nuclei. Accordingly, the asynchronicity in replication between the two nuclei could be explained by the inequal partitioning of a hitherto unidentified essential replication operator/s.

5. With respect to the genes located in regions of HoRT collisions, the enrichment of chromatin dynamics, cell cycle regulation, and DNA replication/repair pathway GO terms are not too surprising. As the only genes that are actively transcribed during the S-Phase would only be these genes and those that are required for Mitosis. This is reiterated by the negative correlation of HoRT conflict zones with the genes for VSP (variant-specific surface proteins). If the whole cell transcriptome data for Giardia during the S-phase is available, it would be interesting to check if

the HoRT collision sites corelate with loci containing highly transcribed genes. It is very likely that they would.

Minor comments:

The authors should discuss regarding the status of DNA replication/cell cycle control in other parasites in the introduction and discussion in the light of their results.

In conclusion, While the paper provides a panorama of useful data pertaining to the curious replication dynamics of Giardia lamblia, it does not clearly define the cause for the asynchrony in replication between its two nuclei. This is not a demerit to the data provided in the paper, but the body of this manuscript does not justify its title; which should be amended to reflect its insight into the broad strokes of replication dynamics occurring within the parasite.

Reviewer 2

Advance summary and potential significance to field

This manuscript describes a comprehensive analysis of genome replication in an interesting non-model eukaryote (Giardia). By combining complementary techniques the authors have been able to gain insight to the nature of DNA replication in this organism. The study will form a valuable platform for further analysis of the cell cycle and DNA replication in Giardia.

Comments for the author

I have a few suggestions that I hope will help the authors improve their manuscript.

Major

1. The authors use an established method to estimate inter-origin distance (IOD) - pulse labelling with different nucleotide analogues followed by DNA combing. In the authors labelling protocol the cells are subjected to two sequential 20 min pulses after which DNA is extracted for analysis. Therefore, the maximum labelling time is 40 minutes (some cells will enter or exit S phase during one of the pulses and therefore be subjected to a shorter labelling time). This compares to the authors estimate of a 70 minute S phase. As such the analogue pulses can only label at most ~50 of genome replication. For example, on a very long combed molecule where the authors detect two replication initiation events hundreds of kb apart (as commonly seen and reported in Fig. 1F) the DNA between the initiation events is yet to replicate and may include origins that activate after the second pulse. A consequence of this is that there may be more (perhaps twice?) origins than detected in the combing data. I think it would be useful for the authors to discuss how the length of the pulse labelling (which is appropriate and standard) relative to S phase duration could influence the calculate IOD and potentially underestimate the number of origins and hence also under estimate the IOD.

2. The double analogue labelling followed by microscopy to visualise replication in the binucleate cells was particularly interesting. Again, I think it is worth the authors considering the consequence of the duration of labelling (in this case two 60 minute pulses) relative to the S phase duration (70 minutes). I also think these data could be presented more clearly by combining some of the categories. From the manuscript, my understanding is that the two nuclei are fundamentally comparable and therefore (for example) the scenarios where just one nuclei is labelled with IdU are equivalent irrespective of whether it is the left or right nuclei that is labelled. I would suggest four cataegories:

A, relatively synchronous replication where both nuclei have the same labelling patterns (as used by the authors);
B, where one nuclei starts replication before the other (one red, one unlabelled nuclei; and the cases of one green and one yellow nuclei);
C, where one nuclei completes replication before the other (one green, one unlabelled nuclei; and the cases of one yellow and one red nuclei);

D, those cases where one nuclei labels but the other does not or labels only with the other analogue.

Of these categories the first three (A-C) are relatively easily understood whereas the final group (D) is harder to explain. I think these categories could aid in presenting the authors data. It would also be worth commenting on some of the differences in labelling observed in Fig 4A. For example, the IdU labelling in row 4 (where one nuclei has just incorporated IdU and the other has just incorporated CldU) looks very different from most of the examples of IdU labelling, although noteworthy the reciprocal pattern in row 12 is also unusual. There are further examples with very limited labelling with one of the labels (rows 5, 6, 9, 12, 15) Are the presented examples typical? I.e. are do all examples in those categories limited labelling of one or both nuclei with one or both labels? Some of these examples hint at different replication dynamics between the nuclei.

3. The authors use a cutting-edge nanopore approach to detect BrdU incorporation on single sequenced molecules. I think it would help readers understand the approach and analysis undertaken if the authors included a figure showing an example molecule and detection of BrdU, the observed BrdU decay and the inferred fork direction.

Minor

- in figure 1 how do the 20 μm scale bars correspond to DNA distances (e.g. in kb).

- in the calculation of the length of cell cycle phrases the authors use the abbreviation ccu - this should be defined somewhere.

- for the origin numbers estimated from combing per chromosome (shown in Fig 3B) the authors should more clearly refer to the appropriate methods section describing how this was determined. It's also unclear why the authors use a y-axis scale up to 40 when all of the data is below 15!

- in the text describing figure 4 I think it would be more appropriate to describe the analogue (I.e. CldU and/or IdU) rather than the applied color. It would also help to switch the column order of the CldU and IdU (panel A)
to reflect that cells where first labelled with IdU and then CldU.

- I was unable to access the sequence data deposited at NCBI SRA.

Reviewer 3

Advance summary and potential significance to field

Giardia is a binucleate parasitic protist, which like all diplomonads possess two equivalent nuclei. It has been known for many years that often the nuclei are slightly asynchronous when dividing as evidence by cytology of spindles or flagellar duplication. The molecular mechanisms for nuclear synchrony or asynchrony remain unknown. The primary claim of this manuscript is that the genome organization causes asynchronous replication between two nuclei (as listed in the title).

The approach used to address this claim is primarily sequence based descriptive data which sought to define structure and timing of replication, progression of replication forks. Nonetheless, the authors present evidence of the use of only several origins of replication and report a higher replication rate than some other parasitic protists. Using BrdU and ErdU labelling the authors also estimate S phase duration. Importantly, the authors report only about 20% asynchrony between the two nuclei.

Comments for the author

The title and thus the primary claim is based on observation of potential head on related to chromatin dynamics, cell cycle regulation, DNA replication, and DNA repair (Figure 5). This claim is problematic as when asynchronous nuclei are only observed about 20% of the time, how could any common mechanisms be associated with genome organization? This discrepancy instead suggests

that other factors are important for cell cycle synchrony and coordinated replication between nuclei beyond genomic organization. Thus, as presented here, the evidence are not in support of primary claims.
It should also be noted that Giardia is a poorly annotated and often-mis-annotated genome, and combined with the divergence of genes, GO annotations are not sufficient for primary conclusions. For example - a common issue is the annotation of ankryin-repeat proteins as "putative spindle-pole proteins" . These are then pulled into the mitosis or cell division category in GO. Because the actually ORF IDs are not presented here (rather just the summary notations form GO) it is not possible to review any of the conclusion from summary figures in Figure 5. Thus, for improved rigor and reproducibility, the authors need to provide the actual ORF ids used for their GO analyses.
Further, all analyses of mitosis are limited to post-mitotic or cytokinesis stages which might also confound the interpretation of (a)synchrony of the nuclei. See https://doi.org/10.1242/jcs.03276 for a clearer interpretation of stages that could be associated with the work presented here.
In summary,the primary concern with the manuscript is less about the descriptions of replication origins and timing, but the strength of the evidence presented in Figure 5 to support the primary claim of nuclear asynchrony deriving from genome organization. One suggestion for improvement and revision, is thus to reframe research questions in a manner that the data support. Or alternatively provide more direct evidence in support of mechanisms of nuclear cell cycle asynchrony.
The latter approach is admittedly beyond the scope of this study, and it remains unclear if a description of timing and number of replication forks merits the high impact for Giardia biology that is suggested in the manuscript.

First revision

Author response to reviewers' comments

Point-by-Point Response to Reviewers

All the authors appreciate the reviewers' opinions. We rewrote parts of the manuscript and changed the weak points raised, trying to follow the suggestions as far as possible. Also, we answered the comments point-by-point, adding information regarding DNA replication/cell cycle control in other protozoans, and comparing them with our results. We also applied the 'one-way ANOVA on ranks' nonparametric test on the data in Figure 4, and replaced the manuscript's title with something more appropriate and consistent with our findings. Modifications are shown in blue in the new version of the manuscript. We hope that these changes have been enough to make our work more robust and informative, leaving it fit for publication in the JCS.

Reviewer comments:

Reviewer #1:

In this paper by M S da Silva et al., the authors investigate the spatio-temporal organization of DNA replication, progression of replication forks, and potential occurrence of head-on replication-transcription collisions in G. lamblia trophozoites using single-molecule techniques, including DNA combing and nanopore-based sequencing. The authors were able to identify caveats of parasite replication including its high replication rate, long inter-origin distance and the parasite's dependence on a relatively small pool of origins within its genome. Additionally, the authors also show that while roughly 80% of trophozoite stage parasites replicate their nuclei synchronously, about 20% show asynchronous replication between their nuclei. Overall, the findings are important to understand the replication dynamics and crosstalk with transcription in G. lamblia.

1. Figure 1 records data that was obtained from biological replicates. However, the histogram in Figure 1.B. showing replication rate frequency does not have error bars. Does this histogram show a sum of all readings obtained from all the replicates? If so, it would be more accurate to show the mean or median values between replicates with error bars.

Reply: We appreciate the reviewer's opinion. The histogram in Figure 1B shows, indeed, a sum of all readings obtained from all the replicates. The mean values with SD (error bars) of the replication rate shown in Figure 1C.

2. It is very interesting to see that about 20% of trophozoites in a population have asynchronous replication between their nuclei. Were these parasites synchronized before the double pulse with the halogenated thymidine analogues? It is not exclusively mentioned anywhere in the manuscript. If not, it would be good to repeat the experiment using cell cycle synchronized parasites. This can be achieved by sorting using FACS or by centrifugal elutriation or chemical arrest-release treatments using nontoxic agents like cycloheximide or low-dose colchicine. Also, the total sample size of 388 cells may not be adequate for a strong statistical correlation. Since this is a qualitative measurement, estimating nuclear patterns in about 1000 cells from three independently synchronized cultures could eliminate bias and provide a more tenable result. Additionally, a non-parametric t-test is required to estimate the significance of this data.

Reply: We partially agree with the reviewer's suggestion. The Giardia trophozoites were not synchronized to perform any of our assays. Usually, synchronization in Giardia is performed using aphidicolin (a specific inhibitor of B-family DNA polymerases) (Poxleitner et al., 2008, doi: 10.1128/EC.00415-07; Reiner et al., 2008, doi: 10.1016/j.ijpara.2007.12.005). Thus, the synchronization using aphidicolin blocks cells in "S" phase, which would impair and introduce bias in our analyses involving DNA replication. Furthermore, there are convincing arguments that no whole-culture 'synchronization' method (using chemical synchronizing agents) has been shown to produce a truly synchronized population of cells rather than a population of cells sharing some singular and particular property within that collection of cells (Cooper, 2019, doi: 10.1111/febs.15050). Therefore, we chose to carry out these analyzes without synchronization. Of note, it is well established that the majority of trophozoites in an asynchronous Giardia culture is in the G2 stage of the cell cycle. Consequently, isolation of trophozoites by cell sorting or counterflow centrifugal elutriation (CCE) results in very low yields of cells in G1/S phases (Horlock-Roberts et al, 2017, doi: 10.1128/mSphere.00384-16), to start a truly synchronized culture.
As suggested by the reviewer, we applied a non-parametric test (One-way ANOVA on ranks) and found a p-value < 0.001 among the different patterns analyzed. We evidenced the statistical analysis in Figure 4 (new version of the manuscript).

3. The title of the paper implies that the organization of the Giardia genome affects the synchronicity of replication between the parasite's nuclei. However, the paper does not provide a clear correlation/causation for this curious phenomenon. Are there more HoRT collisions in one of the nuclei in cells that are asynchronously replicating nuclei? If so, a clear correlation is not established here.

Reply: We fully agree with the reviewer. To avoid misinterpretations, we changed the article's title in this new version of the manuscript. The new title is "Cutting-edge approaches reveal curious features in the DNA replication dynamics in Giardia lamblia".

4. The observations recorded in the paper are under normal healthy conditions. While this is useful, it would be more appropriate to study this under conditions that simulate replication stress. Would the asynchronicity in replication between the two nuclei increase in propensity under nutrient limiting conditions? Would a low dose of DNA damaging agents like Cisplatin or Methyl methanesulfonate exacerbate it ? Would reactive oxide stress from peroxides have an effect on it? In order to justify the title of the manuscript, such conditions might provide some context as to why there is synchronicity in replication between the two nuclei and what can disrupt it.
It would be useful to study this phenomenon in context to the localization and abundance of a replication protein like PCNA or a polymerase or a replication fork licensing/initiating protein. This would help to quantitate the asynchronicity between the two nuclei in terms of the enrichment of these proteins and might help to identify a pathway that regulates synchronicity in replication between the two nuclei. Accordingly, the asynchronicity in replication between the two nuclei could be explained by the unequal partitioning of a hitherto unidentified essential replication operator/s.

Reply: We appreciate the reviewer's suggestion and agree that the peculiar features found by us could be exacerbated in the presence of replication stress or DNA damage caused by genotoxic

agents. However, using cutting-edge techniques, we have already observed such interesting DNA replication features under natural conditions. We believe that it deserves to be published in this form. Especially as this is the first time these features have been evidenced. As mentioned earlier, we changed the article's title in this new version of the manuscript to avoid misinterpretations.

5. With respect to the genes located in regions of HoRT collisions, the enrichment of chromatin dynamics, cell cycle regulation, and DNA replication/repair pathway GO terms are not too surprising. As the only genes that are actively transcribed during the S-Phase would only be these genes and those that are required for Mitosis. This is reiterated by the negative correlation of HoRT conflict zones with the genes for VSP (variant-specific surface proteins). If the whole cell transcriptome data for Giardia during the S-phase is available, it would be interesting to check if the HoRT collision sites correlate with loci containing highly transcribed genes. It is very likely that they would.

Reply: The paragraph was rewritten in order to point out that the obtained GO enrichment was expected. To the best of our knowledge, there is no cell transcriptome data for Giardia during the S-phase available. Nevertheless, we indicated in the new version of the manuscript the data correlation checking that would be done given the S-phase transcriptome availability.

Minor comments:

The authors should discuss the status of DNA replication/cell cycle control in other parasites in the introduction and discussion in the light of their results.
Reply: In the new version of the manuscript, we added more sentences (both in the introduction and in the discussion sections) regarding DNA replication/cell cycle control in other protozoans, comparing them with our results.

In conclusion, While the paper provides a panorama of useful data pertaining to the curious replication dynamics of Giardia lamblia, it does not clearly define the cause for the asynchrony in replication between its two nuclei. This is not a demerit to the data provided in the paper, but the body of this manuscript does not justify its title, which should be amended to reflect its insight into the broad strokes of replication dynamics occurring within the parasite.
Reply: We totally agree. As previously mentioned, in order to avoid these issues raised by the reviewer, we replaced the manuscript title with "Clues on the dynamics of DNA replication replication in Giardia lamblia"

Reviewer #2:

This manuscript describes a comprehensive analysis of genome replication in an interesting non-model eukaryote (Giardia). By combining complementary techniques, the authors have been able to gain insight to the nature of DNA replication in this organism. The study will form a valuable platform for further analysis of the cell cycle and DNA replication in Giardia. I have a few suggestions that I hope will help the authors improve their manuscript.

Major comments:

1. The authors use an established method to estimate inter-origin distance (IOD) - pulse labelling with different nucleotide analogues followed by DNA combing. In the authors labelling protocol the cells are subjected to two sequential 20 min pulses after which DNA is extracted for analysis. Therefore, the maximum labelling time is 40 minutes (some cells will enter or exit S phase during one of the pulses and therefore be subjected to a shorter labelling time). This compares to the authors estimate of a 70 minute S phase. As such, the analogue pulses can only label at most ~50 of genome replication. For example, on a very long combed molecule where the authors detect two replication initiation events hundreds of kb apart (as commonly seen and reported in Fig. 1F) the DNA between the initiation events is yet to replicate and may include origins that activate after the second pulse. A consequence of this is that there may be more (perhaps twice?) origins than detected in the combining data. I think it would be useful for the authors to discuss how the length of the pulse labelling (which is appropriate and standard) relative to S phase duration could influence the calculated IOD and potentially underestimate the number of origins and hence also underestimate the IOD.

Reply: We appreciate the reviewer's observation. To be honest, we don't believe that this approach is underestimating the IODs for mainly two reasons. First, this sequential incorporation of thymidine analogs (double-pulse) was carried out in an asynchronous Giardia population, which means that in our analyses, we have Giardia cells in virtually all of the S phase moments. Also, Giardia multiplication occurs predominantly by longitudinal binary fission, meaning that an exponential (in vitro) Giardia population is a clonal population. Thus, the virtual origins that may be triggered between two spaced origins (e.g.: Fig. 1D), as correctly exemplified by the reviewer, would be possibly detected in our analysis. Second, as calculated in Figure 3, the number of origins estimated by DNA combing (Figure 3B-C) is higher than the minimum, i.e., more than enough to allow the full replication of the whole Giardia chromosomes within the S phase duration. Thus, it would not be a parsimonious hypothesis to assume that a cell would expend energy (ATP) to fire replication origins unnecessarily. Unless, of course, in the presence of replication impairment (that's why we decided to carry out our assays in the most natural conditions possible).

2. The double analogue labelling followed by microscopy to visualise replication in the binucleate cells was particularly interesting. Again, I think it is worth the authors considering the consequence of the duration of labelling (in this case two 60 minute pulses) relative to the S phase duration (70 minutes). I also think these data could be presented more clearly by combining some of the categories. From the manuscript, my understanding is that the two nuclei are fundamentally comparable and therefore (for example) the scenarios where just one nuclei is labelled with IdU are equivalent irrespective of whether it is the left or right nuclei that is labelled. I would suggest four categories:
- A, relatively synchronous replication where both nuclei have the same labelling patterns (as used by the authors);
- B, where one nuclei starts replication before the other (one red, one unlabelled nuclei; and the cases of one green and one yellow nuclei);
- C, where one nuclei completes replication before the other (one green, one unlabelled nuclei; and the cases of one yellow and one red nuclei);
- D, those cases where one nuclei labels but the other does not or labels only with the other analogue.
Of these categories the first three (A-C) are relatively easily understood whereas the final group (D) is harder to explain. I think these categories could aid in presenting the authors data. It would also be worth commenting on some of the differences in labelling observed in Fig 4A. For example, the IdU labelling in row 4 (where one nuclei has just incorporated IdU and the other has just incorporated CldU) looks very different from most of the examples of IdU labelling, although noteworthy the reciprocal pattern in row 12 is also unusual. There are further examples with very limited labeling with one of the labels (rows 5, 6, 9, 12, 15) Are the presented examples typical? I.e. Are all examples in those categories limited labelling of one or both nuclei with one or both labels? Some of these examples hint at different replication dynamics between the nuclei.

Reply: We followed the reviewer's suggestion and added the categories suggested in the new version of the manuscript (Figure 4C). The newly established classification is: nuclei replicate together (yellow, 78.49% ±0.7), undetermined patterns (gray, 7.47% ±3.4), One nucleus (LN or RN) completes replication first (blue, 11.54% ±3.6), and One nucleus (LN or RN) initiates replication first (green, 2.51% ±0.5). Moreover, we expanded the discussion section adding possible scenarios that help to explain the nuclei-labeled pattern observed.

3. The authors use a cutting-edge nanopore approach to detect BrdU incorporation on single sequenced molecules. I think it would help readers understand the approach and analysis undertaken if the authors included a figure showing an example molecule and detection of BrdU, the observed BrdU decay and the inferred fork direction.

Reply: We appreciate the reviewers' suggestion and included a new supplementary figure (Figure S2) showing examples of the DNAscent forksense analysis as bedgraphs of BrdU detection and fork direction probabilities viewed in the Integrative Genome Viewer (IGV).

Minor comments:

- in figure 1 how do the 20 μm scale bars correspond to DNA distances (e.g. in kb).

Reply: According to the manufacturer's instructions (Genomic Vision), the stretching factor of the apparatus (used to stretch DNA onto a coverslip) is constant (1 µm = 2 kb). In the new version of the manuscript (legend of Figure 1), we added a sentence pointing to that.

- in the calculation of the length of cell cycle phrases the authors use the abbreviation ccu - this should be defined somewhere.
Reply: The ccu (cell cycle unit, where one unit corresponds to the doubling time) is defined in the legend of Figure 2. We also added a definition of ccu in the main text.

- for the origin numbers estimated from combing per chromosome (shown in Fig 3B) the authors should more clearly refer to the appropriate methods section describing how this was determined. It's also unclear why the authors use a y-axis scale up to 40 when all of the data is below 15!
Reply: The "origins estimated by DNA combing" are the "average number of origins used during S-phase" (see the topic in material and methods). To avoid misinterpretations, we changed the topic of the material and methods section. The new topic is "Average number of origins used during S-phase estimated by DNA combing". Also, there is no scientific/technical reason for the use of the y-axis scale up to 40 in figure 3B. As the data is perfectly visible in this configuration, we chose to keep this scale to embed the legend into the top of the graph.
- in the text describing figure 4 I think it would be more appropriate to describe the analogue (I.e. CldU and/or IdU) rather than the applied color. It would also help to switch the column order of the CldU and IdU (panel A) to reflect that cells where first labelled with IdU and then CldU.
Reply: In the text describing Figure 4, we added a sentence specifying that the antibodies recognize their respective thymidine analogs (red for IdU and green for CldU). We opted to keep the color classification because we believe it is easier to be interpreted for most readers outside the field. However, we agree with the reviewer about CldU and IdU columns order. In the new figure 4A, we switched the columns CldU and IdU.

- I was unable to access the sequence data deposited at NCBI SRA.
Reply: The sequenced data will be publicly available as soon as the manuscript is published. Meanwhile, reviewers can access it through the following URL:
https://dataview.ncbi.nlm.nih.gov/object/PRJNA701959?reviewer=m5rrhf97de3av6a9fsc59c9crt

Reviewer #3:

Giardia is a binucleate parasitic protist, which like all diplomonads possess two equivalent nuclei. It has been known for many years that often the nuclei are slightly asynchronous when dividing as evidenced by cytology of spindles or flagellar duplication. The molecular mechanisms for nuclear synchrony or asynchrony remain unknown. The primary claim of this manuscript is that the genome organization causes asynchronous replication between two nuclei (as listed in the title). The approach used to address this claim is primarily sequence based descriptive data which sought- to define structure and timing of replication, progression of replication forks. Nonetheless, the authors present evidence of the use of only several origins of replication and report a higher replication rate than some other parasitic protists. Using BrdU and ErdU labelling the authors also estimate S phase duration. Importantly, the authors report only about 20% asynchrony between the two nuclei.

1. The title and thus the primary claim is based on observation of potential head on related to chromatin dynamics, cell cycle regulation, DNA replication, and DNA repair (Figure 5). This claim is problematic as when asynchronous nuclei are only observed about 20% of the time, how could any common mechanisms be associated with genome organization? This discrepancy instead suggests that other factors are important for cell cycle synchrony and coordinated replication between nuclei beyond genomic organization. Thus, as presented here, the evidence are not in support of primary claims.

Reply: To avoid misinterpretations, we changed the article's title in this new version of the manuscript. The new title is "Cutting-edge approaches reveal curious features in the DNA replication dynamics in Giardia lamblia". Also, we expand the discussion section adding possible mechanisms that could explain the ~20% of the Giardia cells exhibiting asynchronous replication between nuclei.

2. It should also be noted that Giardia is a poorly annotated and often-mis-annotated genome, and combined with the divergence of genes, GO annotations are not sufficient for primary conclusions. For example - a common issue is the annotation of ankryin-repeat proteins as "putative spindle-pole proteins". These are then pulled into the mitosis or cell division category in GO. Because the actually ORF IDs are not presented here (rather just the summary notations form GO) it is not possible to review any of the conclusions from summary figures in Figure 5. Thus, for improved rigor and reproducibility, the authors need to provide the actual ORF ids used for their GO analyses.

Reply: The enrichment of GO was carried out based on lists of gene identifiers whose genomic coordinates are known to be in regions annotated as conflict regions between transcription and replication machinery. This list of genes was submitted as supplemental material to the article (Table S1 file). Columns L and M contain the genomic coordinates (start and end) on the chromosome specified in column B of the gene whose ID and strand are found in columns O and N, respectively.

Since the list of ORFs of the genes taken into account was also requested, we obtained the requested list through the intersection between the genomic coordinates. The most current listing of ORFs available on the GiardiaDB website is version 28. However, the mapping carried out used the NCBI genome. Out of 5661 genes in potential conflict regions, 2376 of them had intersection with complete ORFs (the whole ORF is included in the gene sequence).
The intersection list is attached (see file genesIntersectFullOrfs.tsv attached as supplementarymaterial). The header of this list is: Gene_Chrom, Gene_Start, Gene_End, Gene_ID, Gene_Score, Gene_Strand, ORF_Chrom, ORF_Start, ORF_End, ORF_ID, ORF_Score, ORF_Strand and Intersection_length.

3. Further, all analyses of mitosis are limited to post-mitotic or cytokinesis stages which might also confound the interpretation of (a)synchrony of the nuclei. See https://doi.org/10.1242/jcs.03276 for a clearer interpretation of stages that could be associated with the work presented here.

Reply: We appreciate the reviewer's suggestion and agree that a three-dimensional analysis of mitosis and cytokinesis would be much more accurate for estimating the duration of these phases. However, we do not believe that a possible inaccurate measurement of cells in mitosis could confound the interpretation of (a)synchrony of the nuclei. In our view (and according to the data presented in our manuscript – Figure 4), an accurate analysis of mitosis/cytokinesis in the context of our findings would be irrelevant, since the ~20% cells exhibiting asynchronous replication between nuclei was identified precisely only through DNA replication monitoring (S phase).

4. In summary, the primary concern with the manuscript is less about the descriptions of replication origins and timing, but the strength of the evidence presented in Figure 5 to support the primary claim of nuclear asynchrony deriving from genome organization. One suggestion for improvement and revision, is thus to reframe research questions in a manner that the data support. Or alternatively provide more direct evidence in support of mechanisms of nuclear cell cycle asynchrony. The latter approach is admittedly beyond the scope of this study, and it remains unclear if a description of timing and number of replication forks merits the high impact for Giardia biology that is suggested in the manuscript.

Reply: We appreciate the reviewer's suggestion. We reframed the research questions highlighted mainly by the inappropriate title previously chosen. In the new version of the manuscript, we opted to remove attention from Figure 5 and draw attention to the peculiar features found during our analysis using cutting-edge approaches, such as spaced IOD, high replication rate, origins usage close to the minimum, and the ~20% cells exhibiting asynchronous replication between nuclei.

Second decision letter

MS ID#: JOCES/2022/260828

MS TITLE: Clues on the dynamics of DNA replication in Giardia lamblia

AUTHORS: Marcelo S da Silva, Marcela O Vitarelli, Vinvent L Viala, Katherine Tsantarlis, David da Silva Pires, Thiago A Franco, Inacio LMJ de Azevedo, Maria Carolina Elias, and Renata Rosito Tonelli
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have adequately rewritten the manuscript to address the comments. The cutting-edge nanopore approach to detect BrdU incorporation on single sequenced molecules and double analogue labelling followed by microscopy to visualize replication in the binucleate cells are both interesting approaches to characterize the asynchrony in replication. The manuscript is suitable for publication in the JCS.

Comments for the author

Minor comment :
The title mentioned in the comments is “Cutting-edge approaches reveal curious features in the DNA replication dynamics in Giardia lamblia” whereas in the manuscript it is “Clues on the dynamics of DNA replication in Giardia lamblia”. Both titles appropriately define the research questions posed within the manuscript, although the authors need to decide on one for publication.

Reviewer 2

Advance summary and potential significance to field

The authors have satisfactorily address each of my concerns in the revised manuscript. I feel that the

manuscript is now suitable for publication.

Comments for the author

I feel that the manuscript is now suitable for publication.